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14. ABSTRACT

The purpose of this work is to prepare and evaluate heterobivalent conjugates of PSMA/hepsin-binding ligands labeled with optical dyes or radionuclides. The sensitivity and accuracy of prostate cancer diagnosis can be improved by dual-targeting of PSMA and hepsin which are highly overexpressed in advanced and metastatic prostate cancer. In this final year, we prepared several heterobivalent PSMA-hepsin conjugates labeled with optical dyes such as Cy7 and SulfoCy7. One of them, designated as **KB304,** exhibited selective uptake in the PSMA/hepsin-expressing cell lines compared to control cells. In vivo optical imaging study confirmed the accumulation of **KB304** in PSMA-HPN-dual-expressing tumors as compared to PC3/ML tumors which express low level of PSMA and hepsin. From these results, we can conclude that **KB304** can be utilized as a potential lead compound for optimizing PSMA-hepsin heterobivalent conjugates.

15. SUBJECT TERMS
PSMA, Hepsin, Prostate Cancer, Heterobivalency, Molecular Imaging

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1. Introduction.

Prostate cancer (PCa) is the leading cancer in the U.S. population and the second leading cause of cancer death in men. (1) An estimated 233,000 new cases and 29,480 deaths of prostate cancer will occur in the US during 2014, according to the Cancer Facts & Figures 2014 published by American Cancer Society (www.cancer.org). Even if current prostate cancer screening methods with the prostate-specific antigen (PSA) blood test and digital rectal examination (DRE) have advanced significantly for the early diagnosis of patients with PCa, the controversy on the efficacy of PSA testing for reducing PCa deaths is currently still being debated. There have been comprehensive clinical studies whether PSA testing is an efficient biomarker in diagnosing PCa and reducing PCa deaths. Two European studies reported that men who receiving PSA screening can lower the risk of deaths from PCa while the study in US did not show any difference between PSA screening group and non-PSA group statistically. (2-4) Due to the lack of PSA specificity and sensitivity for PCa, there has been an increase of unnecessary biopsies/treatments of what would be benign or indolent disease state. Therefore, there is an urgent dire to explore biomarkers for early and precise detection of PCa, in particular small lesions, i.e., recurrent tumors in the surgical bed, local lymph node invasion and other subtle manifestations of the disease in men. Prostate-specific membrane antigen (PSMA) and hepsin are on the line as clinical biologic markers of PCa due to the fact that they are highly expressed in advanced and metastatic prostate cancer.

PSMA is a type II integral membrane metalloprotease that has abundant and restricted expression on the surface of prostate carcinomas, particularly in androgen-independent, advanced and metastatic disease. (5,6) PSMA possesses the criteria of an ideal target for immunotherapy and diagnosis, i.e., expression primarily restricted to the prostate, abundantly expressed as protein at all stages of the disease, presented at the cell surface but not shed into the circulation, and association with enzymatic or signaling activity. (6) SPECT-CT scan of PCa using ^{111}In-capromab pendetide (Cyt-356, ProstaScint®), an [^{111}In]-labeled monoclonal antibody to PSMA, showed promise in the clinic for identifying metastatic tumors in lymph node. (7, 8)

Because of the important functions of PSMA for PCa, there have been a lot of reports on PSMA-targeted imaging probes which were evaluated in preclinical and clinical studies by means of PET, SPECT, and optical imaging techniques. (9, 10, 11) According to the PSMA crystal structures, the PSMA active site consists of two distinct subpockets, which form a 'glutamate-sensor' S1' site (pharmacophore) and an amphiphilic S1 site (non-pharmacophore). The cylinder-shape ~20Å deep tunnel region exists adjacent to the S1 site and projects toward the hydrophilic surface of the enzyme. (12) The structural freedom by the S1 site provided diverse modifications of PSMA-based imaging probes. In particular, the lysine in the P1 site was utilized as a key scaffold in order to 1) take advantage of the many radiohalogenation methods and radiohalogenated prosthetic groups developed previously for reacting with the ε-amino group of lysine residues, and 2) increase the structural diversity of urea-based PSMA inhibitors. (12, 13, 14)

Hepsin is a type II transmembrane serine protease which is preferentially expressed in neoplastic prostate over benign prostate. (15) In addition, the mRNA level of *hepsin* was elevated in ~ 90% of PCa specimens and was > 10-fold higher in metastatic PCa than in normal prostate or benign prostatic hyperplasia (BPH). Hepsin is composed of 413 amino acids and a large C-terminal residue is located in the extracellular region. The serine protease domain at the C-terminus extracellular part is highly homologous among type II trypsin-like serine proteases. (16) There have been no reports on hepsin inhibitors that have been assessed as potential candidates for *in vivo* molecular imaging studies.

Valency is the number of separate connections that one microscopic entity makes with another. Although originating in the idea of chemical bonding, *e.g.*, oxygen and nitrogen gases

possess double and triple bonds, respectively, in biological terms we refer to the number of binding interactions between two species. In general, higher valency yields higher affinity. In the realm of nanoparticles, valency approach becomes even more important because they can accommodate more than just a few functional groups. Kelly et al. reported the successful detection of prostate cancer at the *in vivo* animal studies using hepsin-targeted multivalent nanoparticles. (17) Moderately-potent peptide (IPLVVPL, 120 nM) conjugated with fluorescent-labeled nanoparticle improved binding affinity/avidity for hepsin and exhibited fluorescent signal *via* FACS by >10-fold higher than the peptide alone. Heterobivalent ligands can be developed by attaching two pharmacophores with an optimal spacer as shown in Figure 1. Recently, there have been two successful reports on the heterobivalent ligands by utilizing PSMA as a target protein. (18, 19) Our hypothesis of the original proposal was to discover novel heterobivalent conjugates to bind to PSMA and hepsin simultaneously. They can be further developed to be more sensitive and potent imaging agents for advanced and metastatic PCa. Due to the overexpression of PSMA and hepsin on the cell surface of metastatic PCa tumors, easy access of bivalent imaging probes to the target sites and high affinity for PCa-cells through heterobivalency can be possible.

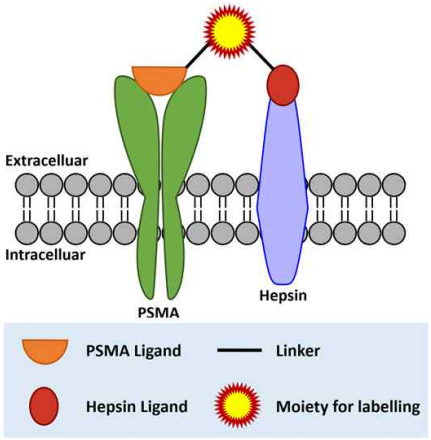


Fig. 1. Design concept of PSMA-Hepsin conjugates

2. Keywords.

Prostate-specific membrane antigen (PSMA), Hepsin, Prostate cancer, Heterobivalency, Molecular imaging

3. Overall Project Summary

3.1. Specific Aims of the project

The sensitivity and accuracy of PCa diagnosis can be improved by dual-targeting of PSMA and hepsin. We attempted to develop heterobivalent conjugates of PSMA/hepsin-binding ligands labeled with optical dyes (Cy 5, Cy7, SulfoCy7 and FITC), positron- or gamma-emitting nuclides, to provide agents of enhanced affinity/avidity for PCa. The PSMA-binding ligand moiety was Lys-urea-Glu, which has been used as the key intermediate for preparation of many PSMA-targeted imaging probes. Hepsin-binding ligand moieties were based on

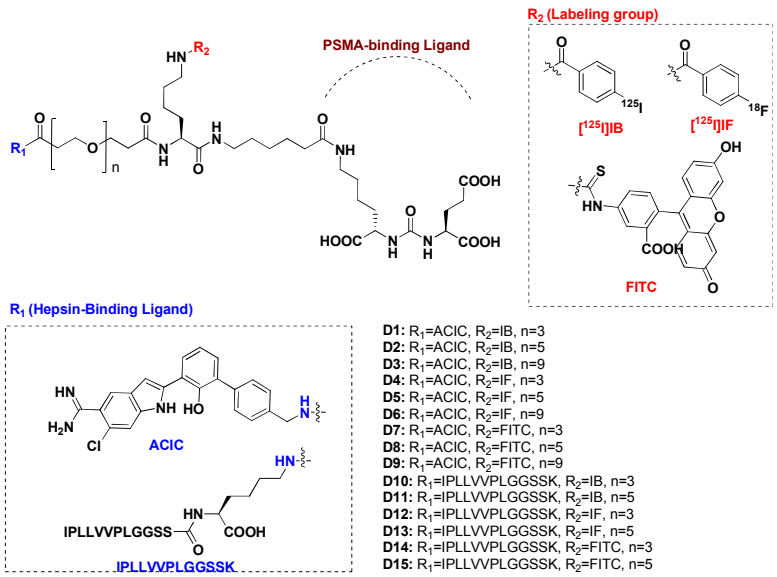


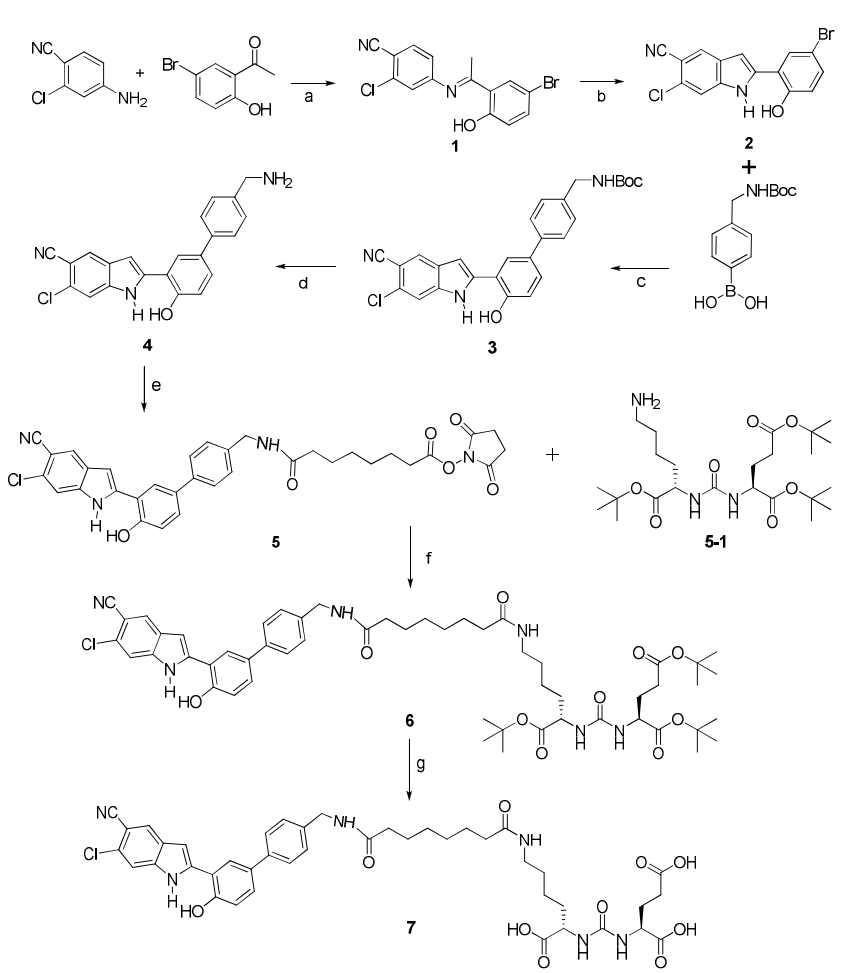
Fig. 2. Examples of designed PSMA-hepsin conjugates

different types of small molecules reported as weak to moderate hepsin inhibitors. (20, 21) These include non-peptide ligands (e.g., indole-5-carboximidamide scaffold) and peptide ligands (e.g., IPLLVVPL) which were identified by phage-display microarrays. (15) The specific aims which we proposed in the original application are as follows.

- ***Aim 1. Synthesis and evaluation of heterobivalent conjugates of PSMA-ligand and nonpeptidic hepsin ligand.*** Goals: Synthesize conjugates of the urea-based PSMA ligand with hepsin ligands derived from indole-5-carboximidamide or thiadiazole with an optimal linker, and evaluate *in vitro* inhibition activities of PSMA and hepsin.
- ***Aim 2. Synthesis and evaluation of heterobivalent conjugates of PSMA-ligand and peptide-derived hepsin ligand.*** Goals: Prepare conjugates of urea-based PSMA ligand with the IPLLVVPL peptide and evaluate *in vitro* inhibition activities of PSMA and hepsin.
- ***Aim 3. In vivo imaging studies of heterobivalent conjugates identified in Aim 1 and 2 with mice implanted with LNCaP and PC3 tumors.*** Goals: Label the heterobivalent conjugates identified in Aim 1 and 2 and carry out *in vivo* small animal imaging studies.

3.2. Progress for Specific Aim 1

3.2.1 Synthesis of PSMA-hepsin conjugates with an indole-5-carboximidamide scaffold



Reagents and conditions: (a) Piperidine, toluene, 120 °C, 25%; (b) $Pd(OAc)_2$, Bu_4NBr (2 eq), DMSO,100 °C, 30%; (c) $Pd(PPh_3)_4$, Cs_2CO_3, DMF/H_2O (5:1), 100 °C, 39%; (d) 25%TFA in DCM; (e) Et_3N, in DMF, 31%; (f) Et_3N, DMF, 21%; (g) 50%TFA in DCM, 43%

Scheme 1. Synthesis of PSMA-hepsin conjugate with indole-5-carbonitrile

Scheme 1 outlines the synthesis of PSMA-hepsin conjugates which has indole-5-nitrile with a suberic acid linker. Preparation of N-aryl imine **1** was achieved by reacting 4-amino-2-chlorobenzonitrile with 5-bromo-2-hydroxyacetophenone under a basic condition (piperidine in toluene) at 120 °C in 25% yield. In order to obtain **1** in higher yield, a variety of reaction conditions were tested by changing temperature, catalyst, and solvent. We tested toluene, *p*-xylene, DMF, and ethanol as reaction solvents, piperidine, triethylamine, $NaHCO_3$, or *p*-TsOH as base or acid catalyst. The highest yield was achieved by utilizing toluene as the solvent and piperidine as the catalyst at 120°C for

6 hr using a Dean-Stark trap apparatus. The low yield during the imine formation step derived from weak nucleophilicity of 4-amino-2-chlorobenzonitrile because of the substitution of two electron-withdrawing groups (CN and Cl) on the aniline ring. When 4-amino-2-chlorobenzonitrile was replaced by aniline under the same reaction condition, the corresponding imine was obtained in 80% yield.

The synthesis of indole **2** was achieved from compound **1** by applying a published palladium-catalyzed oxidative cyclization procedure. (22) Compound **2**, 2-(5-bromo-2-hydroxyphenyl)-6-chloro-1*H*-indole-5-carbonitrile, was obtained in 30% yield with the regioisomer 2-(5-bromo-2-hydroxyphenyl)-4-chloro-1*H*-indole-5-carbonitrile as by-product in 5% yield. To maximize the indole formation, two different reaction conditions were tested: (1) $Pd(OAc)_2$ (10 mol%), Bu_4NBr (2 eq), DMSO, 100 °C, 24 hr, and (2) $Pd(OAc)_2$ (10 mol%), $Cu(OAc)_2$ (3 eq), DMSO, 100 °C, 24 hr. The use of Bu_4NBr underwent the indole cyclization more effectively than $Cu(OAc)_2$ with 2-fold increase of the formation yield. The desired product **2** was separated easily from by-products using normal silica-gel chromatography. The low yield in the indole cyclization step resulted from the steric hindrance by OH group at the *ortho* position of 5-bromo-2-hydroxyacetophenone.

The synthetic step for the preparation of the indole-biphenyl **3** involved the use of palladium-catalyzed Suzuki cross-coupling reaction. The effects of solvent and base on the formation of **3** were evaluated without changing $Pd(PPh_3)_4$ as catalyst. DMF, ethanol, and DMSO were tested as solvent and Na_2CO_3, $NaHCO_3$ and Cs_2CO_3 as base. The highest yield for the preparation of **3** was achieved by the use of Cs_2CO_3 as base and DMF/H_2O (5:1) as solvent at 100°C for 12 hr. The amino group of 4-(aminomethyl)phenylboronic acid should be masked by *tert*-butyloxycarbonyl (*t*-Boc) group for the Suzuki cross-coupling reaction. Use of unprotected 4-(aminomethyl) phenylboronic acid have been plagued with coupling problems and did not afford **3**. Removal of *t*-Boc group from **3** was achieved by the treatment of trifluoroacetic acid (TFA) in dichloromethane (DCM) to give compound **4**. Compound **4** possesses nucleophilic functional group to react with electrophilic moieties. Compound **4** was conjugated with suberic acid bis-(N-hydroxysuccinimide ester) under the basic condition to afford the N-hydroxysuccinimidyl suberate (DSS) ester intermediate **5** in 21% yield. The PSMA-hepsin conjugate **6** was prepared by reacting **5** with *t*-Boc-protected Lys-urea-Glu (**5-1**). Compound **5-1** was prepared from the commercial lysine and glutamic acid in 3 steps in high yield by modifying the reported procedure by us. (12) The *t*-Boc group of **6** was removed by using TFA at room temperature to give the PSMA-hepsin conjugate **7** in 43% yield. The chemical structure of **7** was fully confirmed by ^{1}H NMR and HRLC-MS ([M-H]$^-$: 829.2986) in negative mode as shown in Fig. 3.

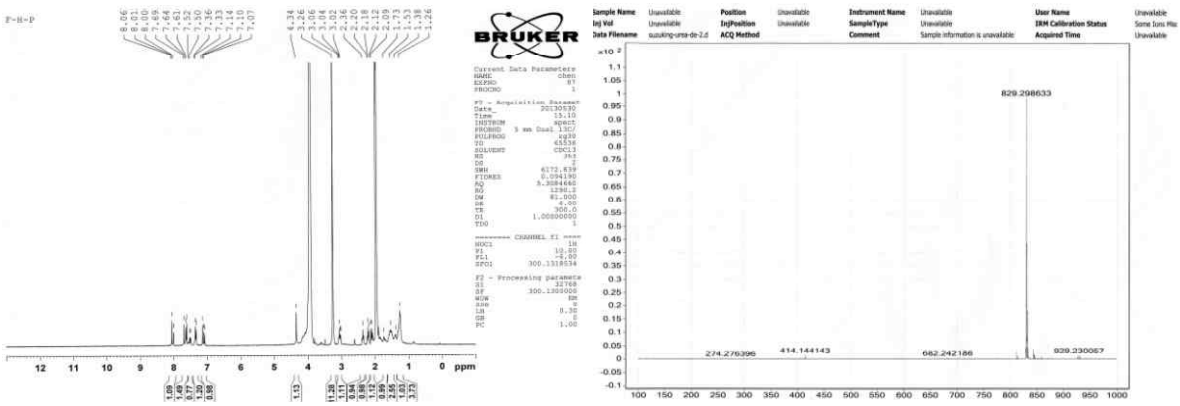
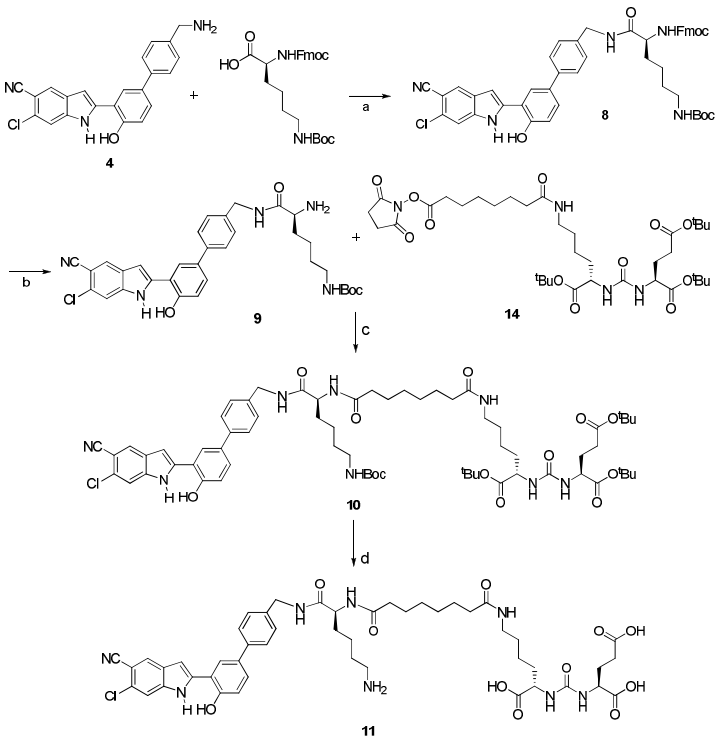


Fig. 3. ^{1}H NMR and HRMS spectra of compound 7

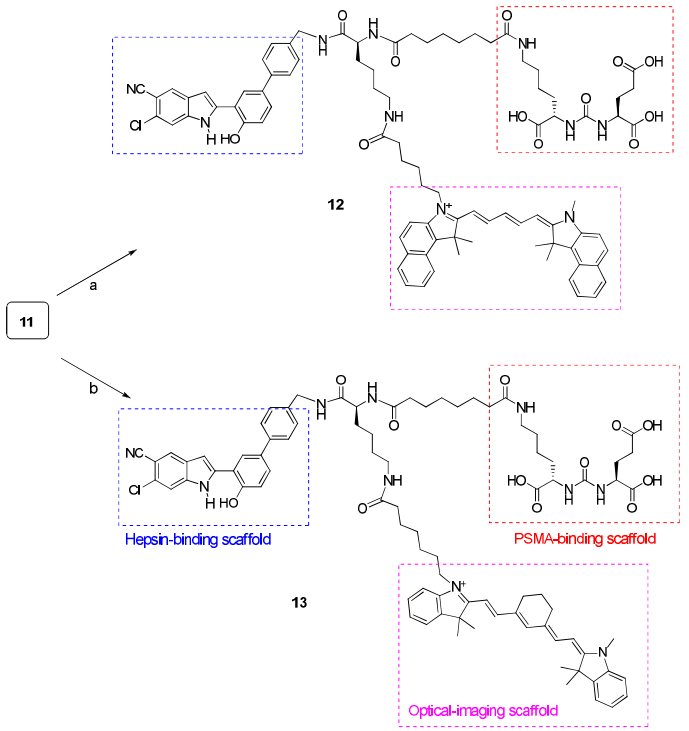
In order to label PSMA-hepsin conjugates with optical dyes, compound **7** had to be further modified to introduce nucleophilic functional groups. Therefore, we synthesized lysine-linked



Scheme 2. Synthesis of the Lys-linked PSMA-hepsin conjugate

PSMA-hepsin conjugate **11** as shown in Scheme 2. The primary amine moiety in **11** can be reacted with a variety of electrophilic optical dyes as well as radiolabled prosthetic groups. Reaction of the indole-biphenyl amine **4** with commercial N_{ε}-*t*-Boc-N_{α}-Fmoc-(L)-lysine under the peptide-coupling conditions (HATU and TEA in DMF) afforded compound **8** in 50% yield. Selective cleavage of Fmoc group of **8** was achieved by the treatment of 25% piperidine in DMF to give compound **9.** Coupling of **9** with compound **14,** which was obtained by reacting excess DSS ester with **5-1**, afforded the PSMA-hepsin conjugate **10** in 27% yield (overall 2 steps). Removal of *t*-Boc and *t*-Bu groups of **10** by the treatment of 50%TFA in DCM afforded the precursor **11** in 40% yield. Primary amine at the side chain of Lys is nucleophilic enough to react with the activated ester moiety of commercial Cy5, Cy7 and SulfoCy7.

Scheme 3 outlines the synthetic route for PSMA/hepsin-targeted near-infrared (NIR) fluorescent agents. The commercially available amine-reactive active esters of Cy5, Cy7 were conjugated with **11** in Tris-HCl buffer (0.1 M, pH=8.5) at room temperature to afford the dye-linked PSMA-hepsin conjugates **12** and **13** in moderate yield. These two final compounds could be purified by reversed-phased HPLC and analyzed by high resolution LC/MS. Purifications of compounds **12** and **13** were achieved by HPLC with gradient method arrayed by 700 nm and 245 nm UV detectors. The optimized HPLC condition for the purification of **12-13** was as follows: mobile phase A (H_2O, 0.5% formic acid), mobile phase B (CH_3CN, 0.5% formic acid), 0 min, 40% B; 10 min, 60% B; 15 min, 40% B. Retention time of **12** and **13** under the same gradient condition



Scheme 3. Synthesis of PSMA-hepsin conjugates with optical dyes Cy5 and Cy7

was 11.1 min and 9.0 min, respectively, indicating that **13** is more hydrophilic than **12**. ESI-MS showed $[M+H]^{2+}$ and $[M+H]^{+}$ ion peaks of **12** and **13** in the positive modes as shown in Figure 4.

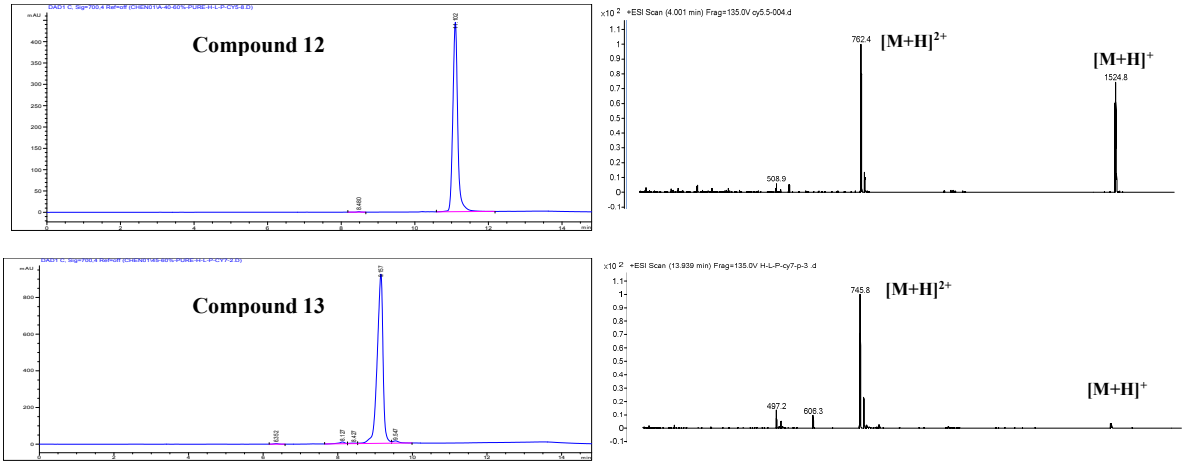


Fig. 4. HPLC chromatograms and HRMS spectra of compounds 12 and 13

The IC_{50} values of the conjugates **12-13** were measured using a fluorescence-based NAALADase assay. As shown in Figure 5, compounds **12** and **13** exhibited moderate inhibitory activities against PSMA *in vitro*. The K_i values of **12** and **13** were 99 nM and 39 nM, respectively. The results indicated that PSMA was well tolerated for the introduction of bulky groups at the P1 site of Lys-urea-Glu ligand when an optimal linker such as suberic acid is attached. However, *in vitro* cell uptake studies of **12-13** exhibited non-specific binding to the PSMA/hepsin-expressing cell lines (The results will be discussed in **3.4.** in more detail).

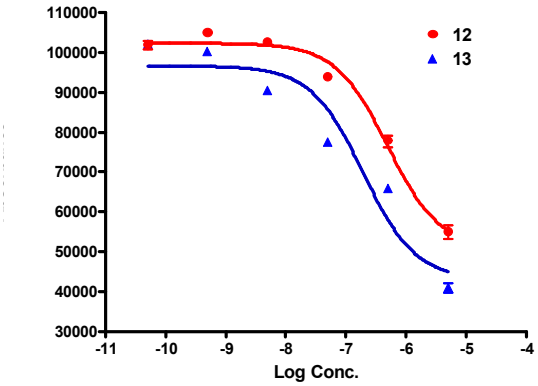
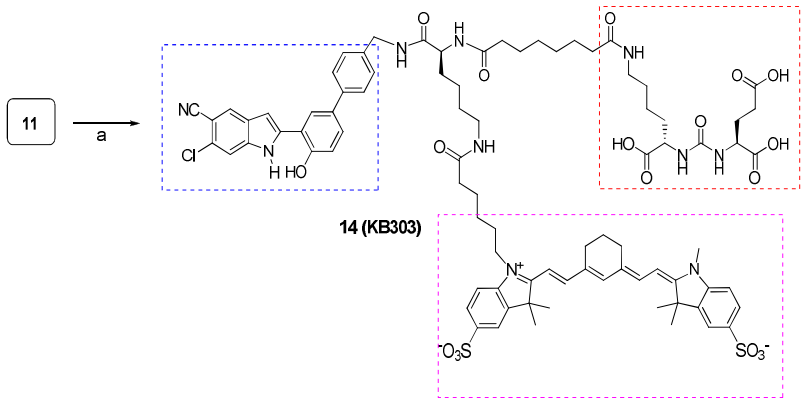


Fig. 5 . IC_{50} curve of compounds 12-13 using a fluorescence-based NAALADase assay

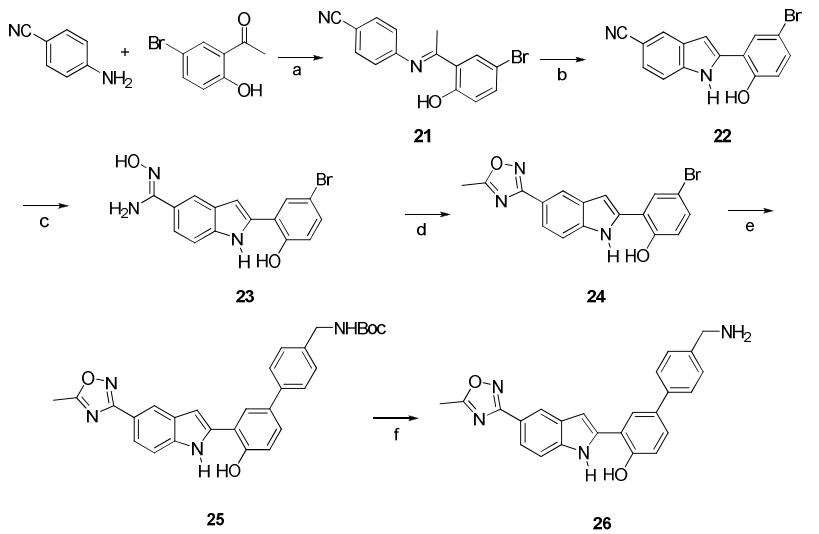
Due to the high lipophilicity of hepsin-binding moiety and optical dyes, **12-13** did not show favorable physicochemical properties at *in vitro* cell uptake studies. Therefore, we attempted to introduce SulfoCy7 instead of Cy7 because two sulfonate groups of SulfoCy7 could increase the hydrophilicity of the PSMA-hepsin conjugates. Compound **14** with SulfoCy7 were synthesized by applying the synthetic route outlined in Scheme 4. Reaction of **11** with SulfoCy7 NHS ester in Tris-HCl buffer afforded compound **14** in 40% yield. Compound **14** (**KB303**) showed improved hydrophilicity as compared to **13** and **14** from the HPLC studies. Furthermore, the K_i value of **14** was 0.78 nM, which was comparable to that of ZJ-43, a potent



Reagents and conditions: (a) Tris-HCl (0.1M, pH=8.5), SulfoCy7, rt, 4h, 40%

Scheme 4. Synthesis of PSMA-hepsin conjugate linked with SulfoCy7

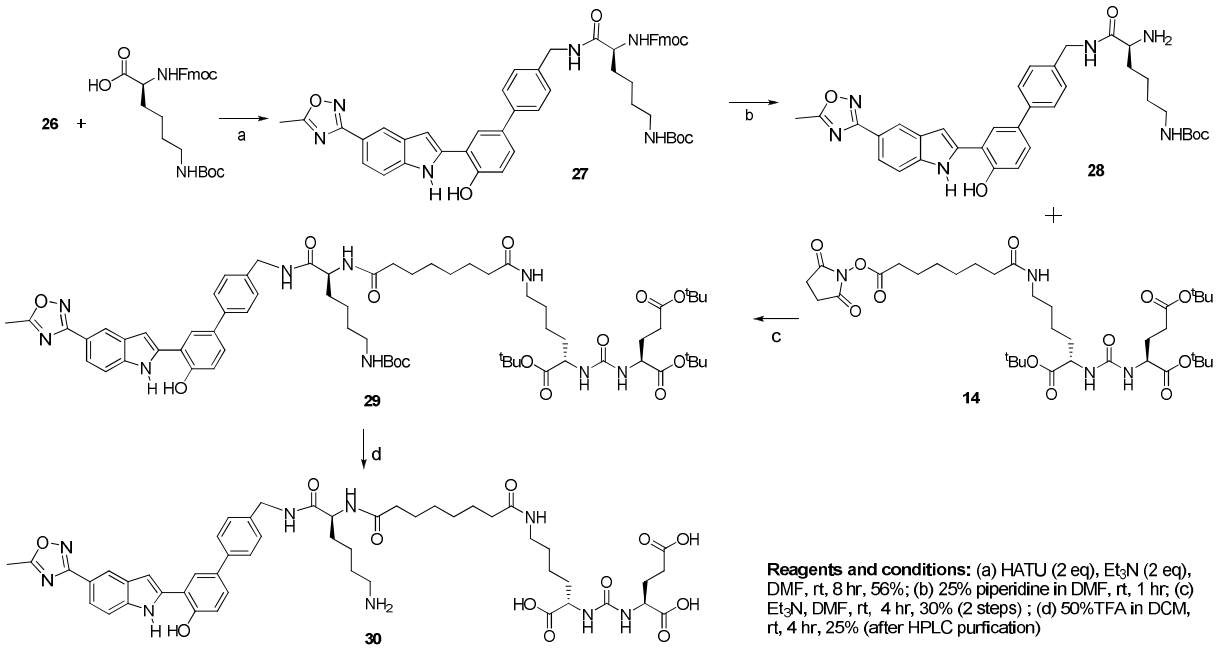
PSMA inhibitor. ZJ-43 showed 0.37 nM of K_i value under the same experiment. As expected, compound **14** exhibited moderate selective-binding to the PSMA-HPN-expressing cell lines at 10 nM concentration (the detailed results will be described in Section 3.4). Even if compound **14** with Sulfo-Cy7 showed selective uptake at 10 nM concentration, it still exhibited non-specific binding to the PSMA-hepsin-expressing cells at high concentration (100 and 1000 nM). The reason why the low-selective uptake of **14** at high concentrations derived from the lack of the chemical scaffold to bind to the hepsin protein efficiently. Initially designed PSMA-hepsin conjugates in the original application contained an amidine moiety on the indole ring of hepsin-binding ligand. The synthesis of PSMA-hepsin conjugates with the amidine moiety was challenging because of the high hydrophilicity of the amidine functional group. Therefore, we utilized the oxadiazole ring as a precursor to afford the amidine group, which would be recovered by hydrogenation reaction. Scheme 5 outlines the synthesis of biphenylindole analog **26** with an oxadiazole ring which would be transformed into the amidine moiety. N-arylimine **21** was obtained by reacting 4-amino-benzonitrile with 5-bromo-2-hydroxyacetophenone under the piperidine-catalyzed condition in 56% yield. The yield of the imine formation step of **21** was improved dramatically as compared to that of compound **1**, probably due to the reduced steric hindrance and the increased nucleophilicity of 4-amino-benzonitrile. This phenomenon was also observed more obviously when aniline was used instead of 4-aminobenzonitrile.



Reagents and conditions: (a) piperidine, toluene, 130 °C, 6 hr, 56%; (b) $Pd(OAc)_2$, Bu_4NBr (2 eq), DMSO,110 °C, 33%; (c) NH_2OH-HCl, Na_2CO_3, 90 °C, 7 hr, 60%; (d) NaOEt, EtOAc, EtOH, 90 °C, 2 hr, 42%; (e) $Pd(PPh_3)_4$, Cs_2CO_3, 4-((*tert*-butoxycarbonylamino)methyl)phenylboronic acid, DMF/H_2O (5:1), 100 °C, 26%; (f) 25%TFA in DCM, 82%

Scheme 5. Synthesis of hepsin-binding ligand with an oxadiazole ring

Intramolecular cyclization reaction of **21** under the palladium-catalyzed oxidative condition gave the indole analog **22** in 33% yield. The nitrile functional group of **22** was converted into the carboximidamide **23** by the treatment of hydroxylamine and sodium carbonate at 90 °C for 7 hours. The carboximidamide group of **23** was transformed into the oxadiazole ring by the treatment of ethylacetate under a basic condition (NaOEt, EtOH) at 90 °C for 2 hours. Compound **24** was obtained from **23** in 42% yield. The conventional palladium-catalyzed Suzuki cross-coupling reaction of **24** with 4-((*tert*-butoxycarbonylamino)methyl)phenylboronic acid to give the biphenylindole **25** in 26% yield. Removal of *t*-Boc group of **25** was achieved by the treatment of TFA in dichloromethane to give 4'-(aminomethyl)-3-(5-(5-methyl-1,2,4-oxadiazol-3-yl)-1*H*-indol-2-yl)biphenyl-4-ol (**26**) in 82% yield.



Scheme 6. Synthesis of the PSMA-hepsin conjugate 30 with an oxadiazole ring

Scheme 6 outlines the synthesis of oxadiazole-protected PSMA-hepsin precursor **30** which can be easily coupled with optical dyes or radionuclide-containing prosthetic groups. Reaction of compound **26** with N_{ε}-Boc-N_{α}-Fmoc-(*L*)-lysine under the traditional peptide-coupling conditions (HATU and TEA) afforded compound **27** in 56% yield. Removal of Fmoc group of **27** was achieved by the treatment of 25% piperidine in DMF to give compound **28.** Conjugation of **28** with the activated DSS ester of PSMA-binding ligand **14** under a basic condition (TEA in DMF) afforded the oxadiazole-containing PSMA-hepsin conjugate **29** in 30% yield (2 steps). Removal of *t*-Boc group of **29** using 50% TFA in DCM at room temperature for 4 hours gave compound **30** in 25% yield after purification by HPLC. The chemical structure of **30** was fully characterized by ^{1}H NMR and HRMS as shown in Figure 6. ESI-MS spectrum of **30** showed $[M-H]^-$ ion peaks at m/z 980.2 in the negative mode. According to the ^{1}H NMR spectrum of **30**, indicative peaks appeared at 4.30 ppm for benzyl CH_2 as singlet, at 2.96 ppm and 2.81 ppm for two N-CH_2 as triplet, and at 2.54 ppm for CH_3 of the oxadiazole ring as singlet.

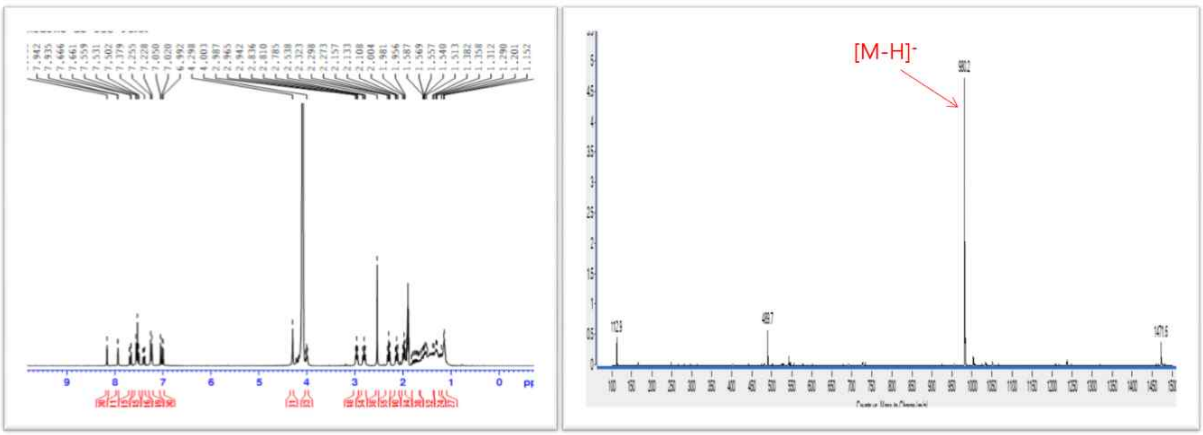
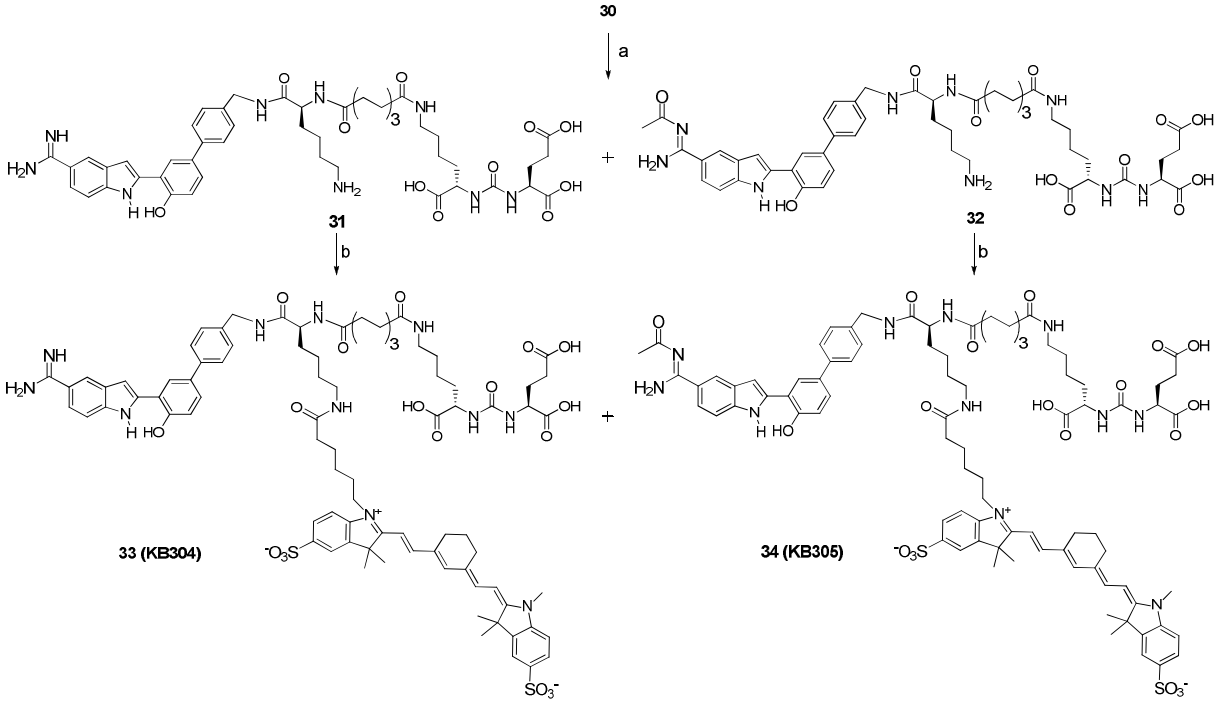


Fig. 6. ^{1}H NMR and HRMS spectra of compound 30



Reagents and conditions: (a) Raney Ni, H_2 (50 psi), dioxane/H_2O/AcOH (6:2:1), rt, 8 hr, 20%; (b) Sulfo-Cy7-NHS ester, Tris-HCl (0.1M, pH=8.5), DMSO, rt, 4 hr, 64%

Scheme 7. Synthesis of PSMA-hepsin conjugates with an amidine moiety

Scheme 7 outlines the synthetic route for the amidine-containing PSMA/hepsin conjugate **34**. The oxadiazole group was deprotected under a hydrogenation condition (50 psi of H_2 and Raney Ni in dioxane) to afford the amidine group. The conversion of the oxadiazole **30** into the amidine **31** was achieved in 20% yield. The low yield of the transformation was due to the fact that the partially-deprotected **32** was obtained as a major by-product. Longer reaction time (> 8 hr) and higher H_2 pressure (> 50 psi) during this step increased the production of the other by-products. Compound **32** was slowly hydrolyzed at room temperature to afford **31**. In order to compare the effect of amidine moiety on the binding to hepsin, compound **32** were also coupled with Sulfo-Cy7. Reaction of **31** and **32** with SulfoCy7-NHS ester in Tris-HCl buffer (0.1 M, pH=8.5) at room temperature for 4 hr afforded the corresponding compounds **33** and **34** in 60-65% yield, respectively. Final compounds **33-34** were purified by reversed-phase HPLC arrayed by 700 nm detector. Although retention time of **33** and **34** were almost overlapped, the complete separation of two compounds could be achieved by using the HPLC gradient method. The optimized HPLC condition for the purification of **33-34** was as follows: mobile phase A (H_2O, 0.5% formic acid),

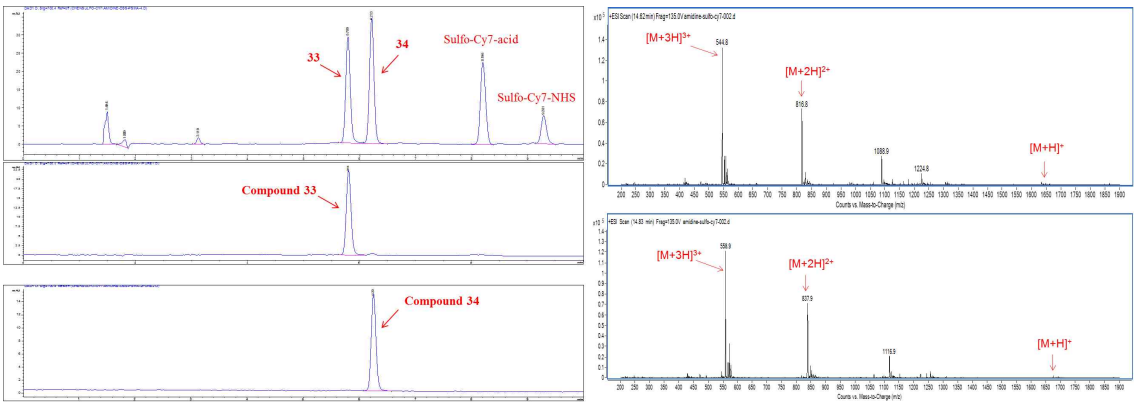


Fig. 7. HPLC chromatograms and HRMS data of compounds 34-35

mobile phase B (CH_3CN, 0.5% formic acid), 0 min, 30% B; 20 min, 40% B; 25 min, 30% B. Compound **33** showed ions with m/z 544.8, 816.8, and 1631.7 as shown in Figure 7. These ions represent $[M+3H]^{3+}$, $[M+2H]^{2+}$, and $[M+H]^{+}$ of **33**, respectively. As shown in Figure 7, compound **34** also showed ESI-MS patterns similar to **33** with $[M+3H]^{3+}$, $[M+2H]^{2+}$, and $[M+H]^{+}$ ions at 558.9, 837.9, and 1673.7, respectively. The retention time of **33** and **34** were 6.8 min and 7.2 min under the optimized HPLC gradient condition at a flow rate of 3.5 mL/min.

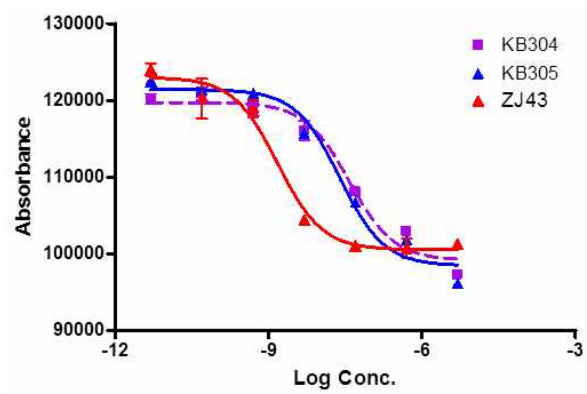
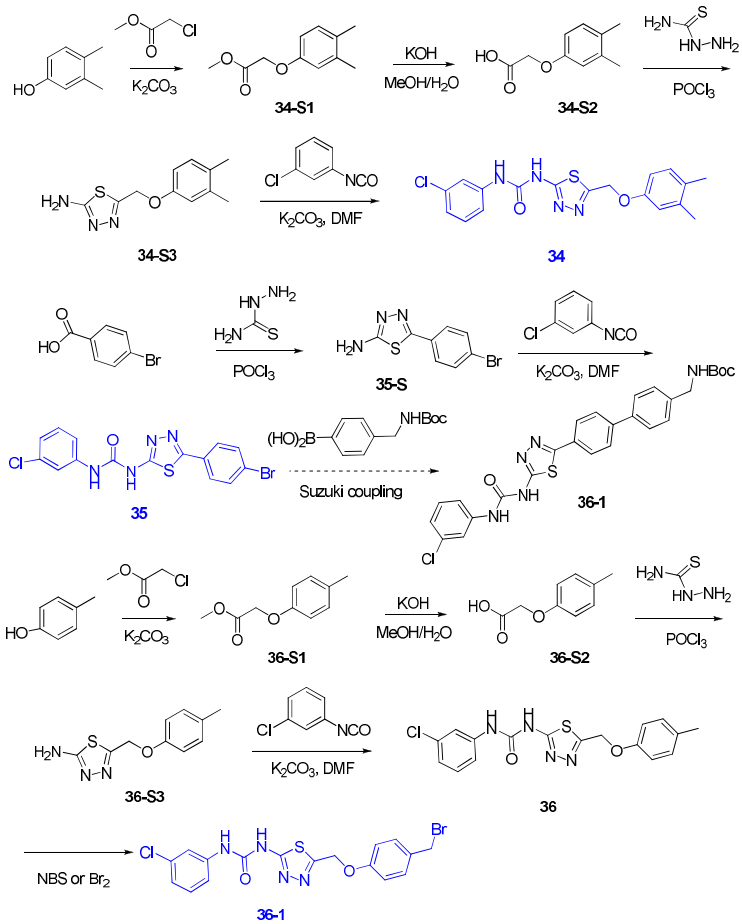


Fig. 8. IC_{50} curves of KB304-KB305

The IC_{50} values of **33-34** were determined by using the fluorescence-based NAALADase assay. As shown in Figure 8, compounds **33** and **34** exhibited strong PSMA inhibitory activities *in vitro*. The K_i values of **33** (**KB304**) and **34** (**KB305**) were 7.6 nM and 4.9 nM, respectively. ZJ-43 showed 0.3 nM of K_i value from the same experiment. These results suggested that PSMA-hepsin conjugates **33-34** with hydrophilic and bulky SulfoCy7 could bind to the active site and tunnel region of PSMA effectively. Specific binding of **KB304** and **KB305** in PC3-PSMA, PC3-HPN, and PC3-PSMA-HPN cells were evaluated in range of 10-1000 nM concentrations (The results will be discussed in 3.4 in more detail).



Scheme 8. Synthesis of hepsin-binding ligands with a diarylurea

3.2.2. Design and synthesis of hepsin-binding ligands with a diarylurea scaffold

The synthetic route for hepsin-binding ligands with a diarylurea scaffold is outlined in Scheme 8. We prepared three hepsin-binding ligands **34-36**. Briefly, 3,4-dimethylphenol was reacted with methyl chloroacetate in DMF in the presence of potassium carbonate at room temperature to give compound **34-S1** in 75% yield. Methylester group of **34-S1** was removed by the treatment of potassium hydroxide in water/methanol/THF (2/2/1) to afford compound **34-S2 2** in 68% yield. Reaction of **34-S2** with hydrazinecarbothioamide in phosphorous oxychloride under reflux provided the thiadiazole **34-S3** in 58% yield. During this step, the reaction mixture should be slowly added to iced water

solution to prevent the explosive reaction of the remaining phosphorus chloride with water (*Caution*). Compound **34-S3** was reacted with 3-chlorophenylisocyanate in the presence of K_2CO_3 in DMF at 125 °C for 24 hr to afford the urea **34** in 55% yield. The synthesis of compound **35** was also performed by applying the method for compound **34** as shown in Scheme 8. Compound **36** was also prepared in a similar way to the synthesis of **34** by using 4-methylphenol instead of 3,4-dimethylphenol as starting material. Treatment of **36** with N-bromosuccinimide (NBS) underwent the radical bromination at the benzylic position to give compound **36-1**. The brominated compound **36-1** could be reacted with incoming nucleophilic groups. *In vitro* IC_{50} of compound **34** with hepsin protein was in the similar range of 0.76 μM. Molecular docking studies of **34** with the hepsin crystal structure using CDOCKER program (DS 3.0, Accelrys Inc.) also exhibited a plausible binding mode of **34** in the active site of hepsin, showing that the urea carbonyl group of **34** is located close to the catalytic site and can make hydrogen-bonding interactions with His 57 (2.26 Å) and Ser 195 (2.63 Å).

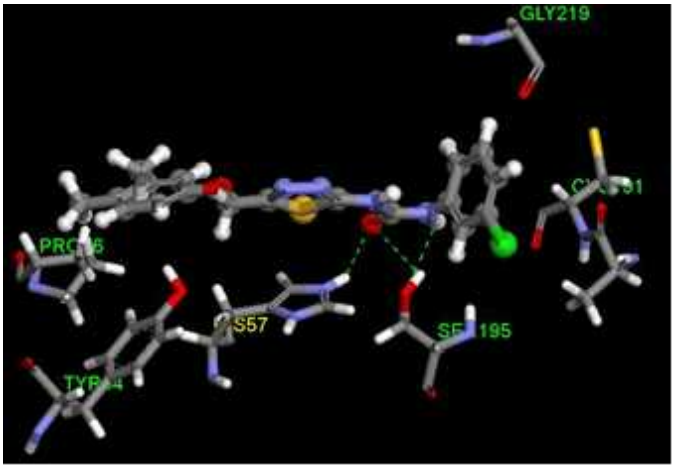
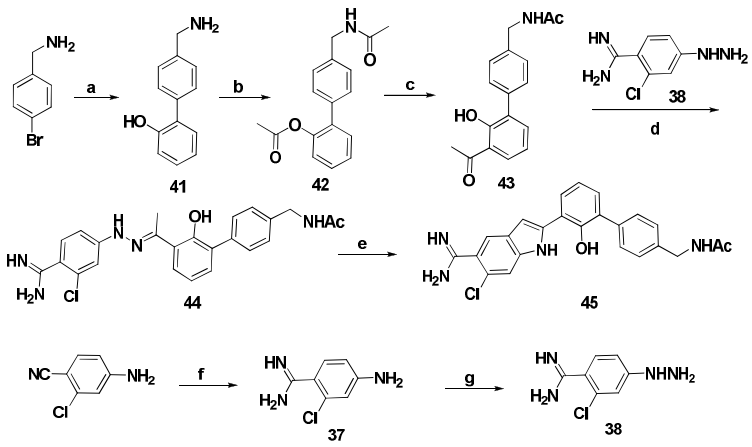


Fig. 9. Binding mode of 34 in the active site of hepsin

The synthetic procedure of the hepsin-binding ligand 2-(4'-(aminomethyl)-2-hydroxybiphenyl-3-yl)-6-chloro-1H-indole-5-carboximidamide, so called ACIC, was outlined in Scheme 9.



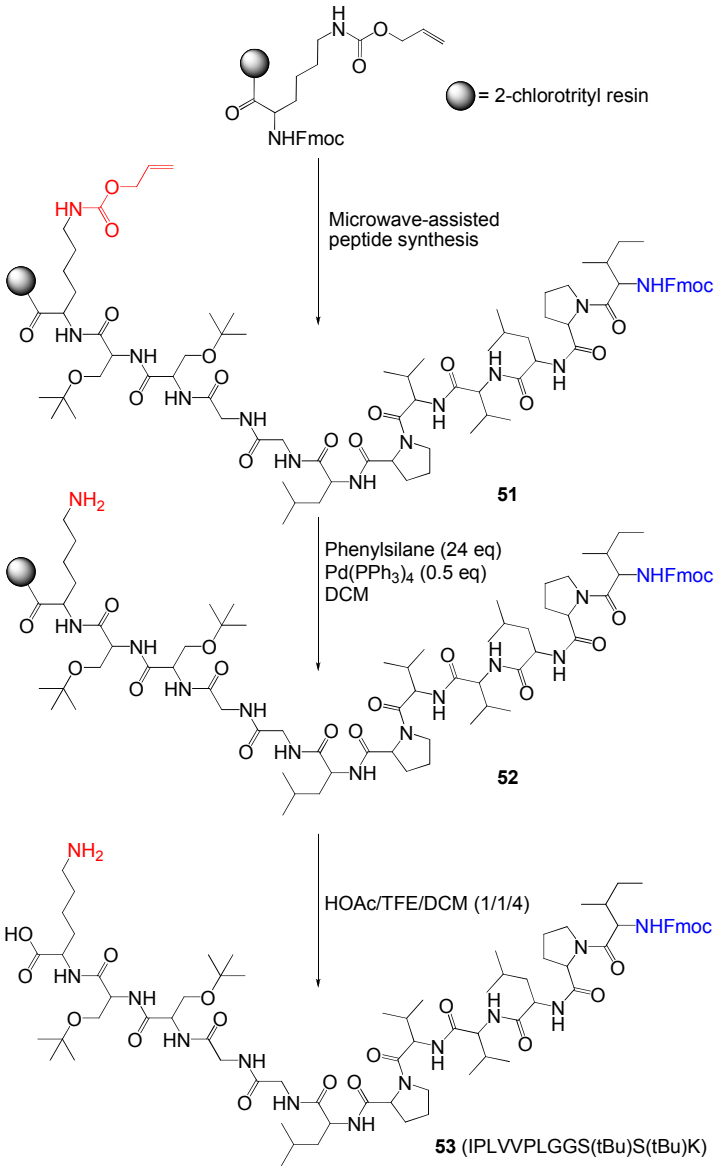
Reagents: (a) 2-hydroxybenzeneboronic acid, Cs_2CO_3, $Pd(PPh_3)_4$, DMF/H_2O, 100 °C, (b) AcCl, pyridine/DCM (1/1), (c) $AlCl_3$ (4 eq), dichlorobenzene, (d) EtOH, TEA, (e) PPA, 155 °C, (f) NH_4Cl, $Al(CH_3)_3$, p-xyelene, reflux, (g) $NaNO_2$, 6N-HCl

Scheme 9. Synthesis of amidine-containing hespin ligands

Suzuki-coupling reaction of 4-bromobenzylamine with 2-hydroxybenzeneboronic acid using $Pd(PPh_3)_4$ as a palladium-catalyst afforded compound **41** in 78% yield. Acetylation of **41** was performed by reacting of **41** with acetyl chloride in pyridine/DCM to give compound **42** in 62% yield. Fries rearrangement of acetyl moiety to the *ortho*-position occurred successfully by reacting **41** with $AlCl_3$ (2 eq) in dichlorobenzene at 130 °C for 24 hr. ^{1}H-NMR studies confirmed that the acetyl moiety of **43** appeared at 2.58 ppm while that of **42** at 2.11 ppm. 2-Chloro-5-hydrazinylbenzimidamide **38** was synthesized from 4-amino-2-chlorobenzonitrile in 2 steps in 35 % yield. Addition of 4-amino-2-chlorobenzonitrile to a mixture of ammonium chloride and trimethylaluminium in *p*-xylene at 0 °C and subsequent stirring under reflux offered the amandine **37**. The amidine was converted into the hydrazine **38** by the treatment of $NaNO_2$ and $SnCl_2$. Condensation of **43** with the hydrazine **38** gave the asymmetric hydrazone **44**. Fisher indolization of **44** mediated by polyphosphoric acid (PPA) afforded **45** in 30% yield.

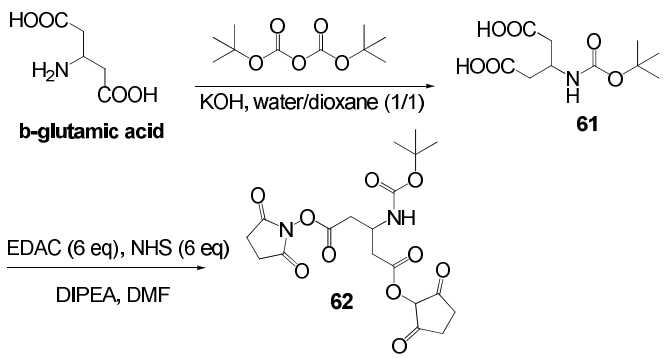
3.3. Progress for Specific Aim 2

3.3.1. Synthesis of hepsin-targeting IPLLVVPLGGSSK



Scheme 10. Synthesis of IPLLVVPLGGSCK peptide

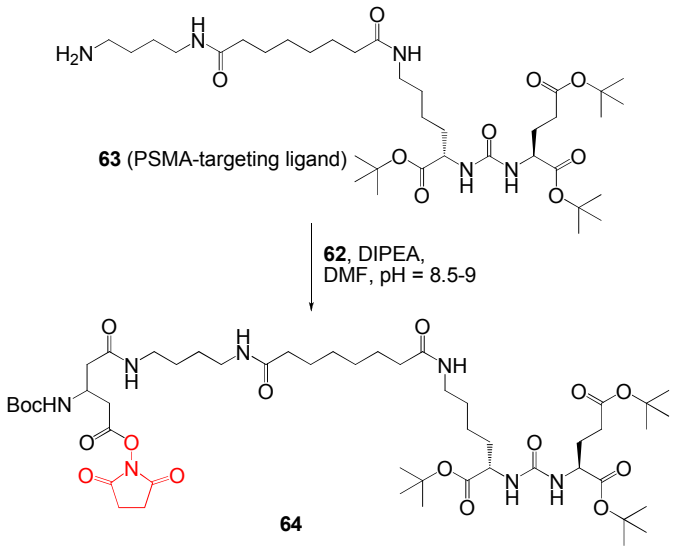
The IPLLVVPLGGSCK peptide, which has an affinity of 190 nM for hepsin-expressing PC3 cells (HPN-PC3 cells), was identified by phage selection method by Kelly et al. (17) According to the report, the conjugates of the IPLLVVPLGGSCK peptide with nanoparticles increased binding affinity for HPN-PC3 cells as compared to the peptide alone. The peptide IPLLVVPLGGS(*t*-Bu)S(*t*-Bu)K was prepared as shown in Scheme 10. Microwave-assisted peptide technology has been successfully applied for the synthesis of IPLLVVPLGGS(*t*-Bu)S(*t*-Bu)K. The allyloxycarbonyl (Alloc) protecting group of C-terminus lysine was used as a starting material. The amine group in the ε-position of lysine was intended to conjugate with the PSMA-binding ligand while the α-position of the lysine or N-terminus isoleucine was linked with imaging prosthetic groups such as optical dyes and radionuclides. The Alloc group was removed by the treatment of phenylsilane and tetrakis(triphenylphosphine)palladium in dichloromethane. Treatment of **52** with a mixture of acetic acid/trifluoroethanol/ dichloromethane (1/1/4) for 2 hr removed the intermediate **53**, IPLLVVPLGGS(tBu)S(tBu)K which was confirmed by ESI-MS, with $[M+H]^+$ ion peak at 1501.8.



Scheme 11. Boc-β-glutamate acid-bis-NHS ester

3.3.2. Synthesis of Boc-β-glutamic acid-bis-NHS ester

The β-glutamic acid shown in Scheme 11 was chosen as a linker of the PSMA/hepsin-targeting moieties as well as a functional group to couple with an imaging probe in order to avoid the formation of structural

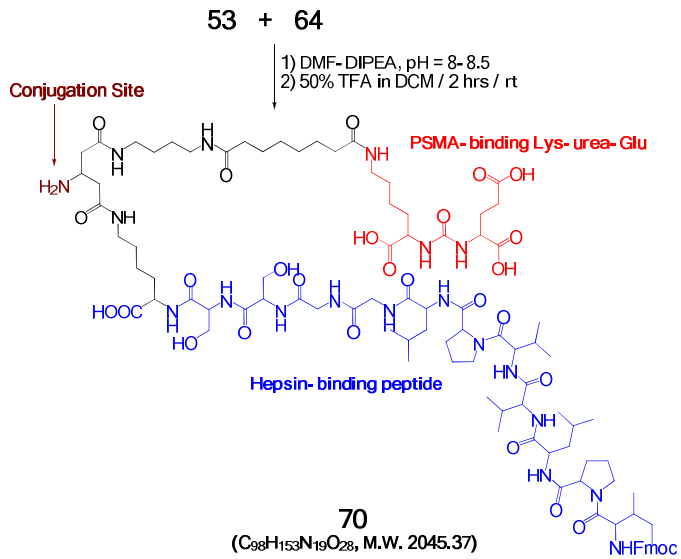


Scheme 12. The synthesis of PSMA-NHS ester

isomers. The two carboxylic acid groups of β-glutamic acid are chemically equivalent whereas those of α-glutamic acid are not. The amine group of β-glutamic acid was protected with *t*-Boc by reacting β-glutamic acid with *t*-Boc anhydride in water/dioxane in 63% yield. NHS-activated ester **62** was synthesized by reacting **61** with NHS using EDAC as a coupling agent in 60% yield. ^{1}H-NMR and ESI-MS confirmed the Boc-β-glutamic acid-bis NHS ester **62**.

3.3.3. Synthesis of PSMA-NHS ester

The PSMA-NHS ester **64** shown in Scheme 12 is a versatile intermediate that can be used to couple with ligands of a variety of target proteins such as hepsin and extracellular metalloproteases. Compound **63** was synthesized by following the procedure we have reported. (12, 14) Conversion of **63** into **64** was successfully achieved under basic conditions (pH: 8.5-9.0) in DMF. Reaction of **63** with 3 equivalents of *t*-Boc-β-glutamic acid-bis-NHS ester **62** afforded **64** in 42% yield without any formation of dimer products. ESI-MS confirmed the formation of **64** with $[M+H]^+$ peak at 1041.2.



Scheme 13. Synthesis of PSMA-hepsin conjugate

3.3.4. Synthesis of PSMA-hepsin conjugate peptide

Conjugation of **53** with **64** was achieved under the basic condition (DIPEA in DMF, pH=8.0-8.5). Subsequent removal of *t*-Bu groups by the he treatment of TFA afforded the target compound **70** which contains PSMA-binding moiety (Lys-urea-Glu) and hepsin-binding moiety (IPLLVVPLGGSSK). ESI-MS confirmed the molecular $[M+H]^+$ ion peak at 2064.4 in the positive mode as shown in Figure 10.

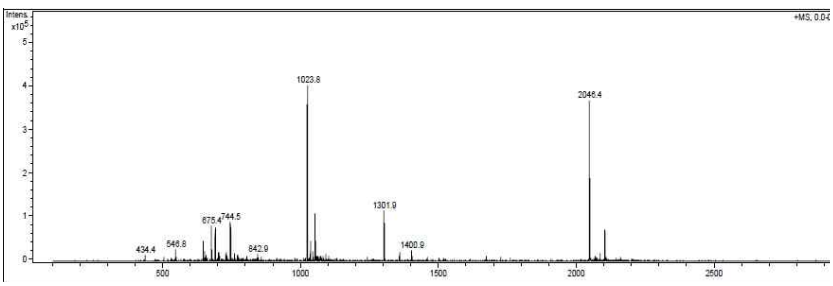


Figure 10. ESI-MS spectrum of compound 70

3.3.5. Synthesis of PSMA-Hepsin conjugates with a DOTA moiety

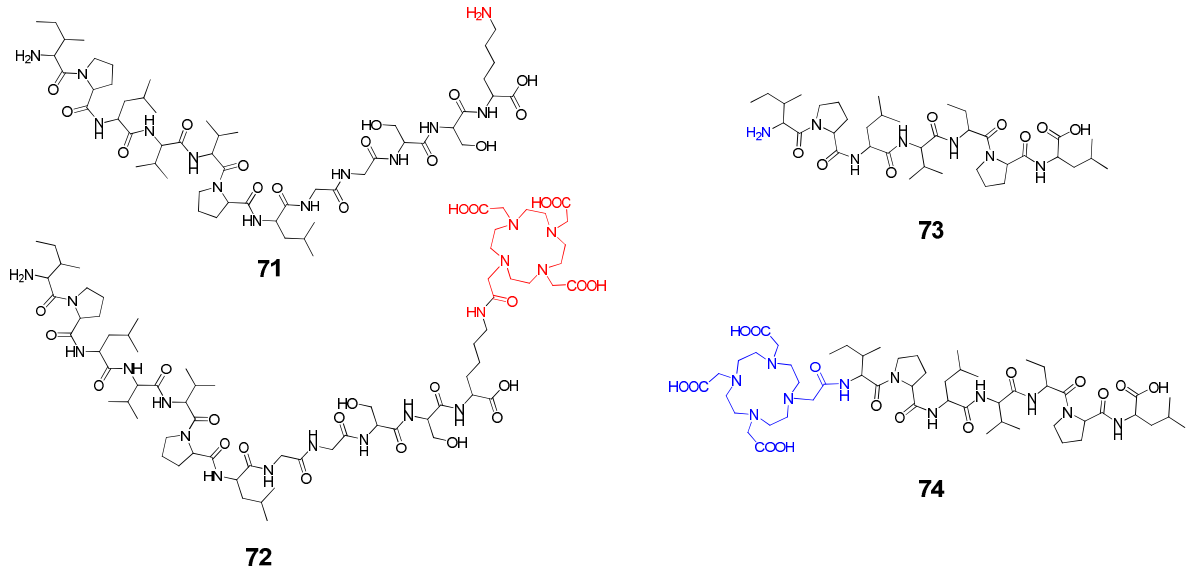


Fig.11. Chemical structures of DOTA-conjugated peptide analogs

The IPLLVVPLGGSSK peptide, which has an affinity of 190 nM for hepsin-expressing PC3 cells (PC3/HPN cells), was identified by phage selection method by Kelly et al. (17). We attached the DOTA moieties to the IPLLVVPLGGSSK (**71**) peptide at the C-terminus and prepared the DOTA-conjugated IPLLVVPLGGSSK peptide **72** (Figure 11). Compound **73** (IPLLVVPL) was also conjugated with DOTA at the N-terminus to afford compound **74** in moderate yield. The compounds **71-74** were purified by HPLC and used for *in vitro* hepsin inhibitory evaluation studies by following the vendor-provided procedure (www.rndsystems.com). The goal of comparing the binding pattern of both N-terminus and C-terminus derivatives was to determine the mode of binding of the IPLVVPL peptide to the hepsin protein and to find the suitable residues for introducing bulky optical dyes or radionuclide-carriers to the peptides. Docking prediction studies of compound **73** with the hepsin crystal structure using ZDOCK module (Discovery Studio 3.1, Accelrys Inc.) showed that the N-terminus of the IPLVVPL peptide projected towards the surface area of the hepsin active site as shown in Figure 12, indicating that the conjugation to the N-terminus of the peptides would be more favorable than C-terminus. *In vitro* hepsin binding affinities of the synthesized peptides were evaluated by the reported assay procedure (www.rndsystems.com). Initial hypothesis was that these analogs would bind to the hepsin active site and would accordingly inhibit the protease activity of hepsin on the known fluorogenic peptide substrate (Boc-Gln-Arg-Arg-AMC). The compounds for *in vitro* assay were prepared by serial dilution from 1 mM to 1 nM and were added to 0.01 μg of recombinant human hepsin (rh hepsin) with the fluorogenic substrate at 1 μM. After incubating the compounds with rh hepsin at 37 °C for 1

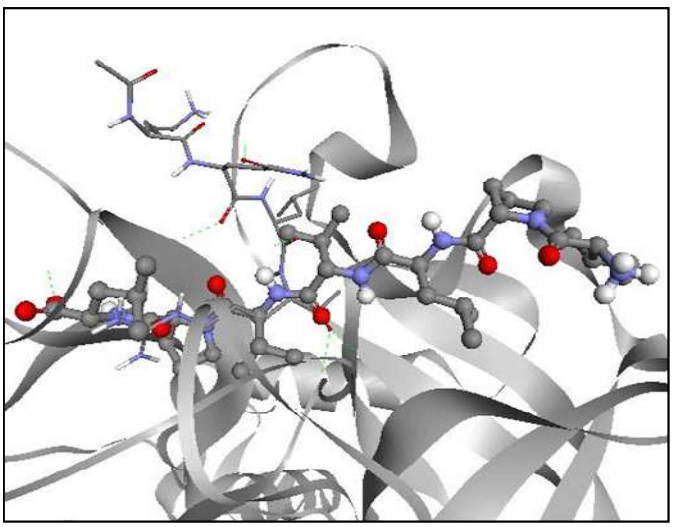


Fig.12. Binding mode of 73 to the active site of hepsin

hour, the fluorescence intensity for each compound was measured at excitation and emission wavelength of 380 nm and 460 nm, respectively. The obtained results exhibited that none of the 4 peptides (**71-74**) inhibit the protease activity of hepsin on its specific fluorogenic substrate. This indicated that these peptides might bind to a hepsin site that is different from the active site of the hepsin protein. In the original publication by Kelly's group (17), IPLVVPL peptide was discovered by a phage-display screen and its binding affinity for hepsin was evaluated with hepsin-overexpressing PC3 cells, not sole hepsin protein.

3.4. Progress for Specific Aim 3

3.4.1. Development of PC3/ML cell lines expressing PSMA and hepsin

We successfully developed the cell lines which express PSMA, hepsin, and PSMA/hepsin. The cell lines can be used for *in vitro* cell uptake studies as well as for generating xenograft mouse models for *in vivo* imaging studies. PC3/ML, a sub-line of PC3 cells with low expression of PSMA and hepsin, was obtained from Dr. Stearns' Lab, Drexel University School of Medicine.

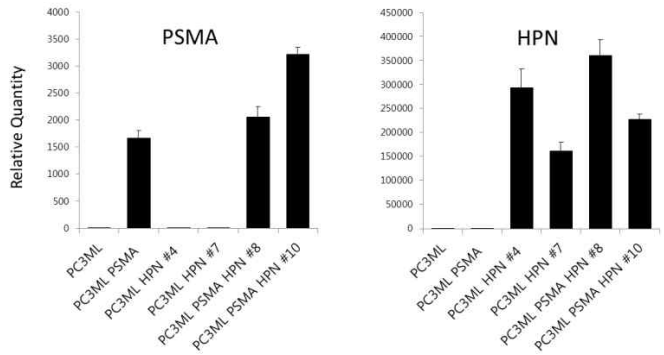


Fig.13. qRT-PCR analyses for PSMA and HPN expression in the developed cell lines

PC3/ML-PSMA cell lines: Full length PSMA cDNA was cloned into pLV vector (lentiviral expression vector) and lentiviral particle were produced by transfecting HEK-293T cells with pLV-PSMA plasmids. PC3/ML cells were transfected with lentiviral particles and the single cell were isolated into 1 well of 96-well plate for clonal expansion using fluorescent activated cell sorter (FACS). Each clone was examined for PSMA expression *via* qRT-PCR and staining with YC-XI-46, the potent PSMA inhibitor conjugated with the Cy5 dye. (23) PSMA-positive clones were selected for generating the cell line. The qRT-PCR result confirmed the PSMA gene expression from the PC3/ML-PSMA cells as shown in Figure 13.

PC3/ML-HPN cell lines: Full length HPN cDNA was cloned into pHIV-Luc vector (lentiviral expression vector for dual expression of fLUC and a gene of interest) and lentiviral particle were produced by transfecting HEK-293T cells with pHIV-Luc-HPN plasmids. PC3/ML cells were transfected with lentiviral particles and the single cell were isolated into 1 well of 96-well plate for clonal expansion using FACS. Each clone was examined for luciferase activity. Two best clones (clone #4 and #7) with luciferase activity were further examined for HPN expression by qRT-PCR. The qRT-PCR result as shown in Figure 13 confirmed the *hepsin* gene expression of PC3/ML-HPN cell lines. The clone #4 was selected for FACS analysis and the cell uptake studies of the synthesized PSMA-hepsin conjugates.

PC3/ML-PSMA-HPN cell lines: PC3/ML-PSMA cell lines were transfected with lentiviral particles (pHIV-Luc-HPN) and the single cell were isolated into 1 well of 96-well plate for clonal expansion. The clones (#8 and #10) with luciferase activity were examined for HPN expression by qRT-PCR. PSMA expression of the clones was further confirmed by qRT-PCR, staining with YC-XI-46, and western blot analysis. The qRT-PCR result (Figure 7) confirmed both PSMA and hepsin gene expression from the #8 and #10 clones of PC3/ML-PSMA-HPN cells. The 8th clone was selected for FACS analysis and the cell uptake studies of compounds.

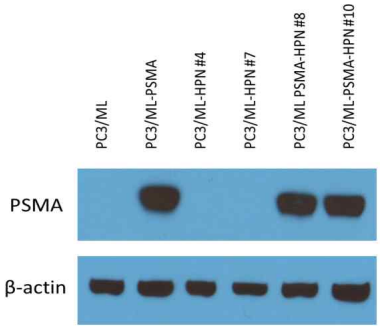


Fig.14. qRT-PCR analyses for PSMA and HPN expression

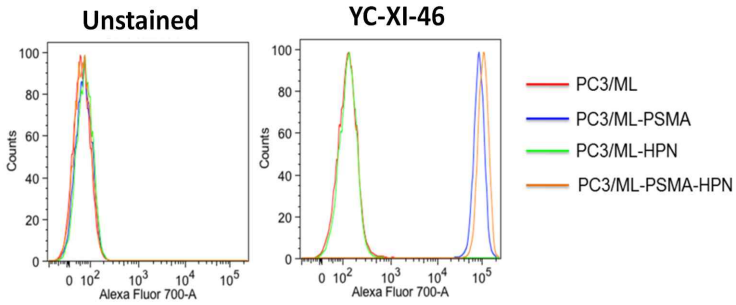


Fig. 15. FACS analysis of the cell lines with YC-XI-46 to confirm the PSMA expression

Western blot analysis of the developed cell lines using the PSMA antibody exhibited the PSMA expression in PC3/ML-PSMA and PC3/ML-PSMA-HPN cell lines as shown in Figure 14. β-actin was used as an internal control for the western blot analysis. The PSMA expression level of the 4 cell lines were evaluated using YC-XI-46 at 100 nM concentration. After staining cells with YC-XI-46, cells were analyzed using FACS (LSRII, BD biosciences). As shown in Figure 15, PC3/ML and PC3/ML-HPN cells remained negative with YC-XI-46 whereas PC3/ML-PSMA and PC3/ML-PSMA-HPN specifically stained with YC-XI-46, indicating the PSMA expression on these two cell lines.

3.4.2. *In vitro* cell uptake studies

Specific binding of the synthesized compounds **12** (**KB301**) and **13** (**KB302**) into the developed cell lines were evaluated at 100 nM concentration. As shown in Figure 16, compound **12** showed the non-specific binding to the three developed cell lines as well as the PC3/ML cell. The lipophilic aromatic rings of the hepsin-binding scaffold in **12** increases the overall hydrophobicity of **12**, thus, resulting in the binding to the cell membranes non-specifically. However, compound **13** with Cy7 is less lipophilic than **12** and exhibited a slightly specific binding for the PC3/ML-PSMA, PC3/ML-HPN, and PC3/ML-PSMA-HPN as compared to PC3/ML, even if the degree of selectivity is low (see the right panel in Figure 16).

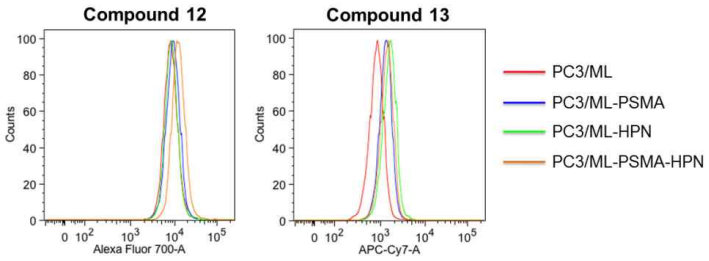


Fig. 16. Binding analyses of 12-13 by FACS labeling methods

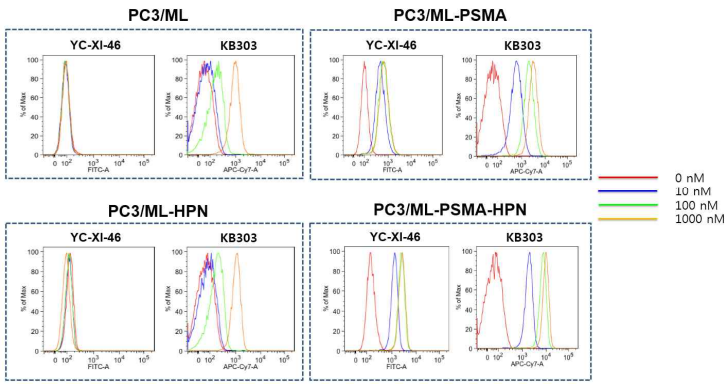


Fig.17. Comparison of cell uptake between YC-XI-46 and KB303

Based on the uptake results of **12-13**, compound **14** (**KB303**) was designed and synthesized. **KB303** contains SulfoCy7 which is much more hydrophilic than Cy7 and Cy5 optical dyes. As shown in Figure 17, **KB303** exhibited improved selective uptake in PC3/ML-PSMA, PC3/ML-HPN, and PC3/ML-PSMA-HPN cell lines. In particular, **KB303** showed the specific binding for PC3/ML-PSMA and PC3/ML-PSMA-HPN cell lines at 10 nM concentration (Figure 17).

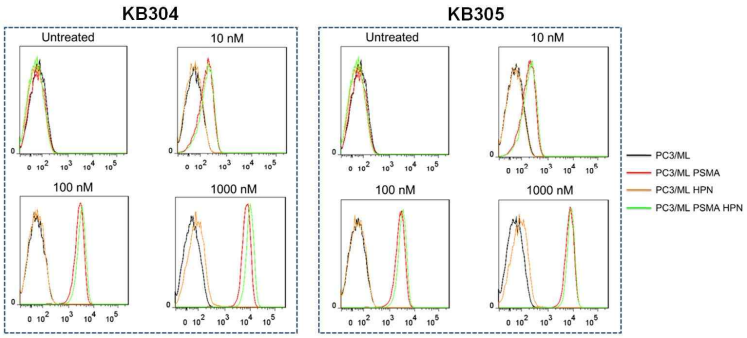


Fig.18. *In vitro* uptake of KB304-KB035 in PC3/ML, PC3/ML-PSMA, PC3/ML-HPN, and PC3/ML-PSMA-HPN cell lines

Encouraged by the result of **KB303**, amidine-containing PSMA-hepsin conjugates **KB-304** and its precursor **KB305** were evaluated. As shown in Figure 18, **KB304** and **KB305** exhibited specific binding to the PC3-PSMA and PC3-PSMA-HPN cells even at the low concentration (10 nM). At high concentrations (100 and 1000 nM), the selective binding of **KB304** and **KB305** to those two cells were observed more obviously as shown in Figure 18. **KB304** and **KB305** also exhibited specific binding slightly for PC3-HPN cell at 1000 nM concentration, suggesting that the amidine-containing PSMA-hepsin conjugates might bind to the extracellular domain of hepsin.

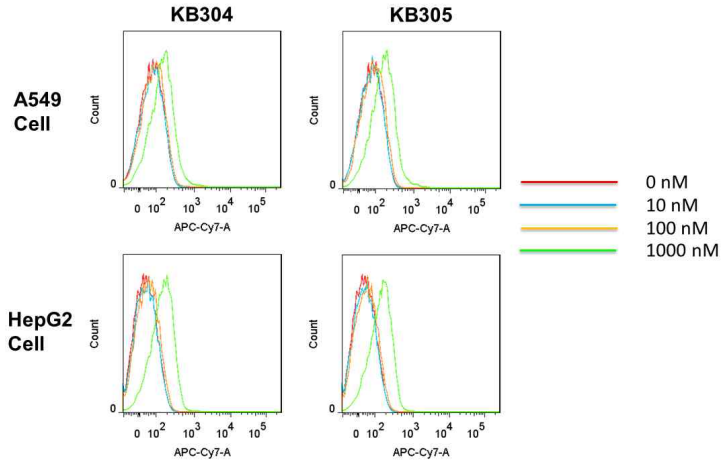


Fig.19. *In vitro* uptake of KB304-KB305 in hepsin-expressing cells

Overall, replacement of nitrile group with amidine increased binding affinity for the hepsin cell lines. To confirm the binding of **KB304** and **KB305** for hepsin cell lines, binding analyses of **KB304** and **KB305** were evaluated using A549 and HepG2 cell lines which express hepsin abundantly. Three different concentrations of those two compounds were tested. Cells were incubated with the compounds for 1 hr at 37 ^{o}C, followed by washing by buffer solution twice. Cells were analyzed by LSRII (BD Bioscience) using APC-Cy7 filter. Both compounds exhibited specific binding at 1000 nM concentration as shown in Figure 19.

3.4.3. *In vivo* optical imaging studies of KB304

Based on the *in vitro* results, **KB304** was selected for *in vivo* NIR optical imaging studies. One million cells were subcutaneously injected into the upper planks of six-week-old NSG (NOD/Shi-*scid*/IL-2Rγ^{null}) male mice. **KB304** (1 nM in 100 μL of PBS) was injected into mice *via* tail vein. Images were taken at 24 hr post injection using the Pearl Impulse Small Animal Imaging System (LiCOR). As shown in Figure 20, **KB304** was accumulated in PSMA-expressing and PSMA-HPN-dual-expressing tumors as compared to PC3/ML tumors which express low level of PSMA and hepsin.

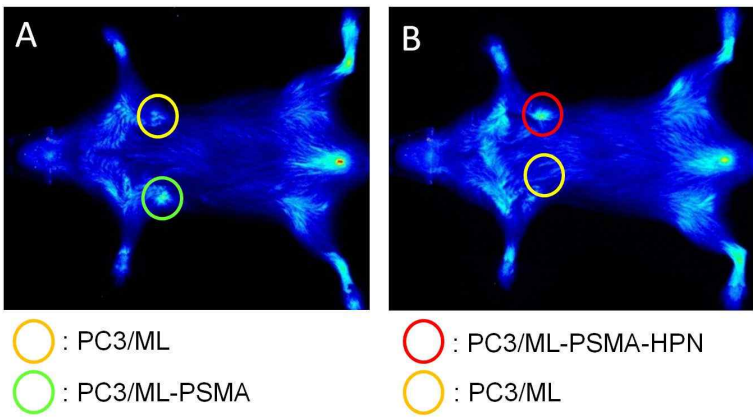


Fig.20. In vivo optical imaging study of KB304 using NSG (NOD/Shi-*scid*/IL-2Rγ^{null}) male mice

4. Key Research Accomplishments

- We established an efficient synthetic strategy to prepare PSMA-hepsin conjugates for structure-activity relationship (SAR) studies. Using the synthetic procedure, we successfully synthesized five novel PSMA-hepsin conjugates labeled with optical dyes (Cy5, Cy7 and SulfoCy7) and evaluated *in vitro* biological activities.
- We also developed the PC3-derived cell lines which express PSMA, hepsin, and PSMA/hepsin. PSMA and hepsin expression on the three cell lines was confirmed by qRT-PCR, FACS, and Western blot analysis. These cell lines were utilized for *in vitro* cell uptake studies and used for generating xenograft mouse models for *in vivo* imaging studies.

5. Conclusion

We have prepared several PSMA-hepsin conjugates labeled with optical dyes such as Cy5, Cy7, and SulfoCy7. The conjugates consist of the PSMA-binding ligand (Lys-urea-Glu), an indole-5-carboximidamide as a hepsin-binding ligand, a suberic acid linker, and the lysine linked with optical dyes. One of them (**KB304**) exhibited strong binding affinity for PSMA and moderate binding for hepsin at *in vitro* enzyme assay studies. It also showed strong selective uptake of PSMA/hepsin-expressing cell lines. We also synthesized the DOTA-linked IPLLVVPLGGSSK-peptides and evaluated their inhibitory activities against hepsin protease. None of them exhibited inhibitory activities against hepsin. Regarding the biology aspect, we successfully developed the cell lines which express hepsin and PSMA/hepsin derived from PC3/ML cells for the *in vitro* cell uptake studies and the *in vivo* imaging studies.

6. Publications, Abstracts, and Presentations

Poster presentation:

Youngjoo Byun, Jianbo Chen, Il Minn, Martin G. Pomper. Design, synthesis and biological evaluation of heterobivalent ligands targeting PSMA and hepsin. *World Molecular Imaging Congress* 2014, Seoul, South Korea, September 17-20, 2014.

7. Inventions, Patents and Licenses

None

8. Reportable Outcomes

None

9. Other Achievements

None

10. References

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11. Appendices

None